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# Neutrophilic inflammation is associated with altered airway hydration in stable asthmatics

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## KEYWORDS

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## Summary

**Background:** Airway dehydration is a potential trigger of bronchoconstriction in exercise-induced asthma; however, its role in stable asthma has not been explored. Using sputum percent solids, as an indicator of airway hydration, we sought relationships between airway hydration and other known markers of neutrophilic (TH1) and allergic (TH2) inflammation in stable asthma.

**Methods:** Thirty-seven atopic subjects with stable asthma and 15 healthy controls underwent sputum induction. Sputum was analyzed for percent solids, cell counts, cellular and biochemical markers of inflammation and purines.

**Results:** Sputum percent solids was significantly elevated in stable asthmatics vs. controls and positively correlated with markers of neutrophilic/TH1-type inflammation (neutrophils, IL-8 and AMP). Sputum percent solids were not correlated with markers of allergic/TH2-type inflammation. These data suggest a direct relationship between neutrophil inflammation and airway hydration in stable asthmatics.

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## Introduction

Acute airway dehydration is reported to be a trigger for bronchoconstriction in exercise-induced asthma.<sup>1,2</sup> Recent studies from both animal models<sup>3</sup> and human subjects<sup>4</sup> suggest that chronic changes in airway hydration may also play an important but under appreciated role in asthma pathophysiology. Chronic airway dehydration is known to contribute to defects in mucociliary clearance in cystic fibrosis, which is also characterized by increased neutrophilic inflammation.<sup>5,6</sup> Like cystic fibrosis, exacerbations of asthma are also associated with decreased mucociliary clearance<sup>7</sup> and acute increases in neutrophilic inflammation.<sup>8</sup> These observations suggest that airway neutrophilia may contribute to dehydration of the airway surface, which, in turn, may result in decreased mucociliary clearance. It has been previously shown that induced sputum selectively samples bronchial airway surface secretions<sup>9</sup> without dilution by fluids recovered from alveolar spaces, and that the percentage of sputum mass which is comprised of solid material (as determined by sputum weight before and after desiccation of the sample and reported as percent solids) is a marker of airway hydration.<sup>4,10,11</sup> As an initial exploration of the relationship between airway neutrophilia and dehydration in asthma, we compared percent solid content of induced sputum in a cohort of subjects with stable asthma with samples recovered from healthy controls. We further assessed the relationships between sputum percent solids and established markers of airways inflammation including granulocytes and cytokines characteristic of neutrophilic ( $Th_1$ ) and allergic ( $Th_2$ ) inflammation. In addition, we measured adenylyl purines, including adenosine triphosphate (ATP) and adenosine (ADO) that act as signaling molecules for purinergic pathways that regulate both inflammatory responses by macrophages and neutrophils and hydration of airway secretions mediated by epithelial cells.<sup>10,12–14</sup>

## Methods

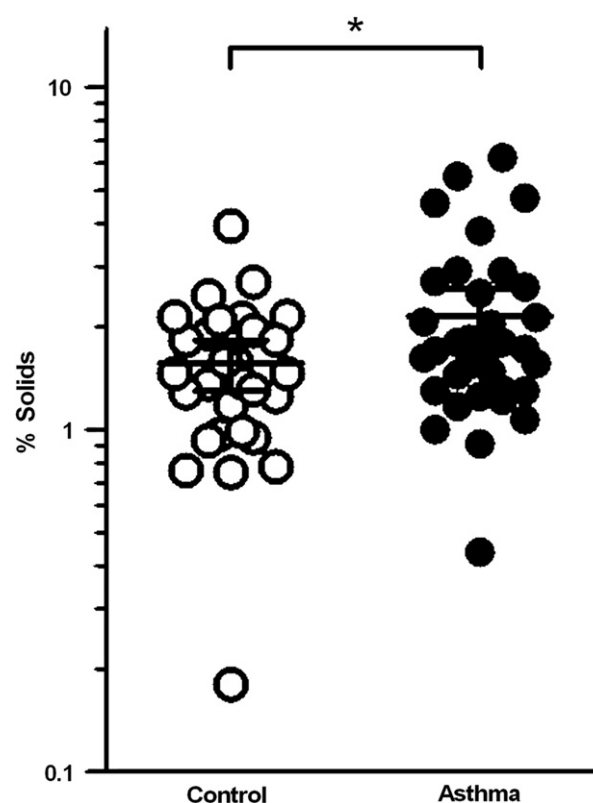
All subjects were non-smoking, 18–50 years of age. Atopic asthmatics ( $n = 37$ ) had physician-diagnosed asthma and were skin test positive to a least one common aeroallergen in our standard skin test panel. The majority of subjects with asthma were in the NHLBI mild category based on  $FEV_1$  (34 mild and 3 moderate persistent), and 14 were on inhaled corticosteroid therapy. Controls were healthy subjects ( $n = 15$ ) and were free of disease. Lung function (mean %predicted  $FEV_1$ ) was modestly lower in subjects with asthma ( $FEV_1 = 98.7\%$  with a range of 74–123%) versus controls ( $FEV_1 = 109\%$ , with a range of 91–121%,  $p < 0.05$ ). During a period of clinical stability, all subjects underwent pulmonary function testing, and sputum was obtained by induction with hypertonic saline as previously described.<sup>15</sup> In brief, sputum plug material was selected by visual inspection of the sample where identifiable plugs (more dense/opaque material vs. clear watery saliva) were manually retrieved using sterile pointed forceps. Sputum percent solids were assessed as a measure of airway hydration status by measurement of pre- and post-desiccation weights of selected sputum plugs (200–500 mg),<sup>4,11</sup>

and total and differential leukocyte counts on sputum were obtained from processing the remaining plug material.<sup>15</sup> Sputum supernatants were obtained as previously described<sup>15</sup> and analyzed for IL-1 $\beta$ , IL-8, IL-4, IL-5, and IL-13 in the asthma cohort (using Luminex-based cytokine assays<sup>15</sup>) and for adenylyl purines AMP, ATP, ADO, ADP (using etheno-derivatization and HPLC<sup>16</sup>) in all subjects.

For statistical analysis, sputum measures did not follow a normal distribution. Therefore, all sputum measures were log transformed to normalize the distribution (transformed data passed KS normality test). Student's unpaired parametric  $t$ -test was utilized for between group comparisons and an overall level of  $p \leq 0.05$  was considered to be statistically significant. All values were expressed as the mean  $\pm$  SEM. Correlation analysis to test for the degree of a linear relationship between two variables was performed using Pearson's correlation coefficient ( $r$ ), where  $p < 0.05$  was considered a statistically significant correlation. GraphPad Prism V.3.1 statistical software (GraphPad Software) was used for all statistical analyses.

## Results

We measured the percent solids content in sputum and observed it to be significantly elevated in asthmatics compared to sputum recovered from controls (Fig. 1). To explore whether airway inflammation was related to airway hydration, we examined the correlations of sputum percent



**Figure 1** The percent of total weight of sputum samples (95% confidence intervals) which is comprised of solid matter is increased in subjects with stable asthma (2.2%) as compared to normal subjects (1.6%).

**Table 1** Subject demographics and sputum characteristics.

		Healthy controls	Asthma
Subject demographics	<i>n</i> =	15	37
	Age	27 ± 5.3	30 ± 9.5
	FEV <sub>1</sub> (% predicted)	108.8 ± 9.4	98.7 ± 14.2*
Sputum cell counts	<i>n</i> =	13	33
	TCC/mg	1052 ± 664	1389 ± 2638
	PMN/mg	236 ± 147	769 ± 2359
	Macrophages/mg	797 ± 565	603 ± 577
	Lymphocytes/mg	6 ± 5	13 ± 32
	Eosinophils/mg	1.2 ± 2	24 ± 62*
	Epithelial cells/mg	11 ± 18	22 ± 29

solids to inflammatory cell counts and fluid phase markers of inflammation (IL-1 $\beta$ , IL-8, IL-4, IL-5, and IL-13). These cytokines were chosen as representatives of the Th<sub>1</sub> and Th<sub>2</sub> pathways which are important in asthma. Sputum inflammatory cell counts were similar between groups with the exception of increased sputum eosinophils in subjects with atopic asthma (Table 1). In asthmatics, sputum percent solids were significantly correlated with neutrophil counts and IL-8, markers of Th<sub>1</sub> inflammation (see Table 2). Markers of Th<sub>2</sub> inflammation such as eosinophils, IL-4, IL-5 and IL-13 were not correlated with percent solids (Table 3). These findings held true even when the asthma group was sub-divided into those on inhaled corticosteroid (ICS) therapy and those who were not taking ICS.

To determine the relationship between airway hydration and purinergic signaling, we measured adenylyl purines in sputum supernatant. Consistent with previous studies,<sup>17</sup> we observed a significant increase in levels of sputum ADO in asthmatics compared to healthy controls (Fig. 2). No other purines were statistically different in asthmatics relative to controls, though mean concentrations were higher in asthmatics. Furthermore, we did not observe a significant difference between purine levels of subjects with asthma on inhaled corticosteroids (ICS) and those not in ICS therapy. Sputum percent solids were significantly correlated with AMP and to a lesser extent ADO (Table 2). AMP was also correlated with markers of Th<sub>1</sub> inflammation including neutrophils ( $r = 0.5$ ,  $p = 0.003$ ), IL-8 ( $r = 0.6$ ,  $p = 0.001$ ), and IL-1 $\beta$  ( $r = 0.9$ ,  $p = 0.02$ ) (Table 2). In contrast, AMP levels did not correlate with markers of Th<sub>2</sub> inflammation including eosinophils ( $r = -0.15$ ,  $p = 0.43$ ), IL-4 ( $r = -0.03$ ,  $p = 0.91$ ), IL-5 ( $r = 0.2$ ,  $p = 0.7$ ), and IL-13 ( $r = 0.3$ ,  $p = 0.4$ ) (Table 3).

## Discussion

Acute dehydration of airway mucus during exercise has been identified as an important aspect of exercise-induced bronchospasm<sup>1,2</sup> and has been reported in acute exacerbations of asthma and mortality of asthma.<sup>11</sup> In this study, we found that airway hydration was also reduced in clinically stable asthmatics at baseline. While the differences were modest, these results demonstrate that neutrophilic inflammation is associated with airway dehydration in asthmatics. We hypothesize that in asthmatics confronted

with stimuli which are established causes of asthma exacerbation and are also associated with acute neutrophil influx, such as viral infection, pollutant exposures and even exercise, there will be even greater dehydration with loss of mucociliary clearance. While bronchoconstriction is the most immediate feature of airway pathophysiology in asthma, increased mucus secretion and loss of mucociliary clearance are also central features of asthma exacerbation.<sup>7</sup> We hypothesize that airway dehydration will result in both bronchoconstriction and decreased mucociliary clearance.

This hypothesis is supported by recent experimental observations both in animals and humans which demonstrate that chronic changes in airway hydration can worsen bronchoconstrictive responses. Deletion of the aquaporin 5 water channel gene in mice results in reduced airway hydration and a greater sensitivity to bronchoconstrictor challenge agents.<sup>3</sup> A recent study in humans demonstrated a negative correlation between fluid secretion rates in tears and sweat<sup>18</sup> and sensitivity to methacholine-induced bronchoconstriction. These data suggested that individuals who have difficulty rehydrating airway secretions after a dehydrating exercise challenge may be at greater risk for subsequent bronchoconstriction. Though we did not assess fluid secretion rates in this study, our finding of a significant decrease in airway hydration at baseline is consistent with this hypothesis.

**Table 2** Correlations of percent solids with specific sputum endpoints in subjects with asthma.

	Pearson's <i>r</i> -value	<i>p</i> -value
PMN/mg ( <i>n</i> = 31)	0.5	0.001
TCC/mg ( <i>n</i> = 32)	0.5	0.004
IL-8 ( <i>n</i> = 32)	0.7	<0.0001
IL-1 $\beta$ ( <i>n</i> = 7)	0.7	0.07
Adenosine ( <i>n</i> = 27)	0.4	0.07
AMP ( <i>n</i> = 27)	0.8	<0.0001
ADP ( <i>n</i> = 27)	0.1	0.56
ATP ( <i>n</i> = 27)	0.3	0.16

PMN: polymorphonuclear leukocytes; TCC: total cell count; AMP: adenosine monophosphate; ADP: adenosine diphosphate; and ATP: adenosine triphosphate.

**Table 3** Comparison of TH1 and TH2 correlations with percent solids.

	Pearson's <i>r</i>	<i>p</i> -value
TH1 Inflammatory markers		
PMN/mg ( <i>n</i> = 31)	0.5	0.001
IL-8 ( <i>n</i> = 32)	0.7	<0.001
TH2 Inflammatory markers		
Eosinophils/mg ( <i>n</i> = 31)	0.1	0.6
IL-4 ( <i>n</i> = 15)	−0.4	0.1
IL-5 ( <i>n</i> = 7)	0.1	ns
IL-13 ( <i>n</i> = 10)	0.6	ns

While neutrophil counts did not differ significantly between asthmatic and control volunteers, there were significant correlations between percent solids of induced sputum and neutrophil counts and with other markers associated with Th<sub>1</sub> inflammation, including IL-8. Interestingly, the purine with the strongest correlation with sputum percent solids was AMP. This purine has been shown to correlate with neutrophilic or Th<sub>1</sub> inflammation<sup>16,19</sup> and activated neutrophils are known to accumulate both extracellular ATP and AMP.<sup>13,14</sup> Thus all of the measured markers that correlate with sputum percent solids are related to Th<sub>1</sub> inflammatory pathways. In other respiratory diseases such as cystic fibrosis, neutrophilic inflammation has been shown to strongly correlate with ATP and AMP levels in bronchoalveolar lavage fluid of these patients.<sup>16</sup>

In contrast, sputum percent solids were not correlated with any markers of Th<sub>2</sub> inflammation despite the fact that increased Th<sub>2</sub> inflammation is thought to be involved in the majority of individuals with asthma and sputum eosinophils were elevated in our asthmatic cohort compared to controls. These findings suggest that airway dehydration may predominantly affect asthmatics with more neutrophilic inflammation which may relate to the fact that neutrophilic asthmatics generally have more severe disease.<sup>20</sup> Elevated percent solids content in sputum has been reported in other respiratory diseases like cystic fibrosis,<sup>5,10,12</sup> bronchiectasis,<sup>21</sup> and primary ciliary dyskinesia<sup>22</sup> where neutrophils play a dominant role in the airway. Our study suggests that in the airway of subjects

with asthma, even modest variations in neutrophilic inflammation can influence airway hydration and play a more important role in asthma pathophysiology than previously suspected.

Although we observed an increase in adenosine in the asthmatic subjects, as previously reported in bronchoalveolar lavage fluid,<sup>17</sup> the correlation between adenosine and airway hydration was relatively weak. This lack of correlation is not surprising, however, as purinergic signaling via ADO increases chloride secretion to the apical surface of the epithelium and would be expected to increase hydration of mucus on airways surface. Adenosine may form part of a feedback regulatory loop, where hypertonic conditions trigger the release of ATP and its subsequent metabolism to adenosine improve airways hydration.

The importance of airway dehydration in disease exacerbation in asthma is suggested by studies in which administration of nebulized hypertonic solutions that affect the osmolality of airway surface liquid (and increase airway surface hydration) are shown to improve mucociliary clearance in asthma. Daviskas et al.<sup>4,23</sup> showed that subjects with asthma have improved mucociliary clearance after treatment with hypertonic saline and mannitol. We have likewise found that mucociliary clearance is improved in asthmatics after use of hypertonic saline to facilitate mucus clearance from the airway.<sup>24</sup> These studies demonstrate that targeting hydration may be beneficial to asthmatics and build a case for its clinical relevance. Certainly hydration therapies have proven successful in other respiratory diseases. They are currently being used in patients with cystic fibrosis and investigated in patients with COPD. Our data suggests this may be important in asthma as well.

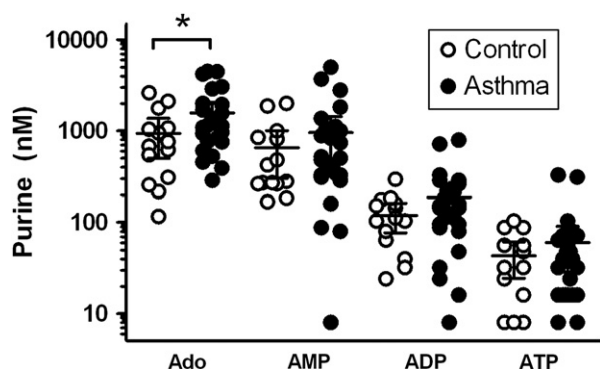
In summary we report an elevated percent solids in clinically stable asthma compared to controls. Further investigation revealed an intriguing relationship between airway hydration and neutrophilic inflammation in the airways of stable asthmatics. Although our data do not necessarily indicate a causal relationship, they highlight the role of neutrophilic inflammation in mediating hydration of asthmatic airways. We suggest that future studies of experimental or naturally occurring asthma exacerbation should account for changes in airway hydration and the role that acute inflammation may have in this process.

## Conflict of interest statement

D.B. Peden has consulting arrangements with GlaxoSmithKline, Novartis, Funxional Therapeutics, and Genentech; and has received grant support from GlaxoSmithKline. The rest of the authors have no declarations or conflicts of interest.

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**Figure 2** Adenyl purine levels (95% confidence intervals) in sputum samples from asthmatic volunteers (solid bars) were elevated compared to those from normal volunteers (open bars), with this difference being significant for adenosine.

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